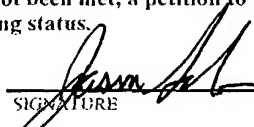


3607 Rec'd PCT/PTO 08 MAR 2002

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|---|--|---|--|--|--|
| FORM PTO-1300 (REV. 11-2000) | | U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE | | ATTORNEY'S DOCKET NUMBER 109312.122US1 | |
| TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371 | | | | U.S. APPLICATION NO. (If known see 37 CFR 1.5) 10/070667 | |
| INTERNATIONAL APPLICATION NO. PCT/GB00/03450 | | INTERNATIONAL FILING DATE 8 September 2000 | | PRIORITY DATE CLAIMED 8 September 1999 | |
| TITLE OF INVENTION SELECTION SYSTEM | | | | | |
| APPLICANT(S) FOR DO/EO/US HOLLIGER et al. | | | | | |
| Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information: | | | | | |
| 1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371. 3. <input type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below. 4. <input checked="" type="checkbox"/> The US has been elected by the expiration of 19 months from the priority date (Article 31). 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau). b. <input checked="" type="checkbox"/> has been communicated by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input type="checkbox"/> An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)). a. <input type="checkbox"/> is attached hereto. b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau). b. <input type="checkbox"/> have been communicated by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)). 9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). | | | | | |
| Items 11 to 20 below concern document(s) or information included: | | | | | |
| 11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. 14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 15. <input type="checkbox"/> A substitute specification. 16. <input type="checkbox"/> A change of power of attorney and/or address letter. 17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825. 18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4). 19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4). 20. <input checked="" type="checkbox"/> Other items or information: | | | | | |
| Copy of International Application No. PCT/GB00/03450 Copy of Amended Claim Sheets submitted in Chapter II Postcard | | | | | |

| | | | |
|---|--------------|--|-----------|
| U.S. APPLICATION NO. 10/070667 INTERNATIONAL APPLICATION NO. PCT/GB00/03450 | | ATTORNEY'S DOCKET NUMBER 109312.122US1 | |
| 21. <input checked="" type="checkbox"/> The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO. \$1000.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00 International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT = | | CALCULATIONS PTO USE ONLY \$ 860.00 | |
| Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)). | | \$ | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE |
| Total claims | 27 - 20 = | 7 | x \$18.00 |
| Independent claims | 3 - 3 = | 0 | x \$80.00 |
| MULTIPLE DEPENDENT CLAIM(S) (if applicable) | | + \$270.00 | |
| TOTAL OF ABOVE CALCULATIONS = | | \$ 986.00 | |
| <input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. | | \$ | |
| SUBTOTAL = | | \$ 493.00 | |
| Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)). | | \$ | |
| TOTAL NATIONAL FEE = | | \$ 493.00 | |
| Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property + | | \$ | |
| TOTAL FEES ENCLOSED = | | \$ 493.00 | |
| | | Amount to be refunded: | \$ |
| | | charged: | \$ |
| a. <input type="checkbox"/> A check in the amount of \$ _____ to cover the above fees is enclosed. b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>08-0219</u> in the amount of \$ <u>493.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>08-0219</u> . A duplicate copy of this sheet is enclosed. d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038. | | | |
| NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status. | | | |
| SEND ALL CORRESPONDENCE TO Henry N. Wixon, Esq. Hale and Dorr LLP 1455 Pennsylvania Ave. Suite 1000 Washington, DC 20004 | | SIGNATURE  Jason J. Schwartz NAME 43,910 REGISTRATION NUMBER MARCH 8, 2002 | |

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | |
|---------------------------------------|---|-----------------------------------|
| In re Application of: HOLLIGER et al. | : | Attorney Docket No. 109312.122US1 |
| Serial No.: Not yet assigned | : | |
| (National Stage of PCT/GB00/03450, | : | |
| Filed Sep. 8, 2000) | : | Group Art Unit: Not yet assigned |
| Filed: March 8, 2002 | : | Examiner: Not yet assigned |
| For: SELECTION SYSTEM | : | |

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Please consider the following Preliminary Amendment which is being filed concurrently with the above-referenced application. The Commissioner is hereby authorized to charge any additional fees which may be required for consideration of this Amendment, or credit any overpayment to deposit account no. 08-0219.

IN THE CLAIMS:

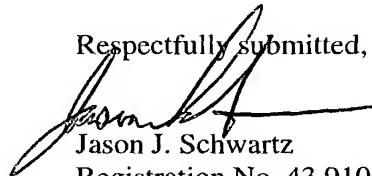
Please amend claims 10, 14-16, 18, 21-23, 25 and 27 as outlined in Appendix 1. A copy of the amended claims showing the changes made is included herein as Appendix 1. A copy of the entire set of pending claims after entry of this amendment is provided herein as Appendix 2.

REMARKS

Entry of the above amendments and examination of the application are respectfully requested. Claims 1-27 are pending. Claims 10, 14-16, 18, 21-23, 25 and 27 have been amended to remove their multiple dependencies. No new matter has been added by these amendments.

Entry of this amendment and favorable examination of the application are respectfully requested.

Respectfully submitted,



Jason J. Schwartz
Registration No. 43,910

HALE AND DORR LLP
1455 Pennsylvania Avenue, N.W.
Washington, DC 20004-1008
(202) 942-8400
Date: March 8, 2002

APPENDIX 1

1. A method for detecting a protein-nucleic acid interaction between a nucleic acid molecule and a protein molecule, comprising the steps of:
 - a) providing one or more hybrid $\sigma 54$ activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active $\sigma 54$ transcription activating domain;
 - b) providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for $\sigma 54$ RNA polymerase (RNAP), which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain; and
 - c) detecting expression of the reporter gene.
2. A method according to claim 1, comprising providing a repertoire of hybrid $\sigma 54$ activator proteins, said repertoire comprising a plurality of different nucleic acid binding sequences.
3. A method according to claim 1, comprising providing a repertoire of hybrid nucleic acid molecules, said repertoire comprising a plurality of different binding sites for the nucleic acid binding sequence.
4. A method according to claim 1, comprising providing both a repertoire according to claim 2 and a repertoire according to claim 3.
5. A method for detecting a protein-protein interaction, comprising the steps of:
 - a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;
 - b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active $\sigma 54$ transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a hybrid protein comprising both a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain; and

e) detecting expression of the reporter gene.

6. A method according to claim 5, comprising providing a repertoire of first hybrid proteins, said repertoire comprising a plurality of bait polypeptides.

7. A method according to claim 5, comprising providing a repertoire of second hybrid proteins, said repertoire comprising a plurality of prey polypeptides.

8. A method according to claim 5, comprising providing a repertoire of first hybrid proteins and a repertoire of second hybrid proteins, said repertoires comprising a plurality of bait and prey polypeptides.

9. A method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

b) providing a $\sigma 54$ activator protein comprising a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the repertoire and $\sigma 54$ activator together with the nucleic acid molecule in a IHF⁻ host cell, such that $\sigma 54$ activator and the nucleic acid molecule may interact, and transcription activated from the $\sigma 54$ RNAP binding site in a manner dependent on DNA bending by the polypeptide factors; and

e) detecting expression of the reporter gene.

10. (Amended) A method according to claim[s 1 to 8] 5, wherein the polypeptides are obtained by expression within a bacterial host cell.

11. A method according to claim 10, wherein the polypeptides are encoded one or more libraries of nucleic acid vectors.

12. A method according to claim 11, wherein a first library of nucleic acid vectors encodes a first chimeric gene, said gene comprising a nucleic acid sequence that encodes a nucleic-binding domain and a nucleic acid sequence encoding a first (bait) test protein or protein fragment in such a manner that the first test protein is expressed as part of a hybrid protein with the nucleic acid-binding domain.

13. A method according to claim 11, wherein a second library of nucleic acid vectors encodes a second chimeric gene, said gene comprising a nucleic acid sequence that encodes a $\sigma 54$ transcriptional activation domain and a second (prey) test protein or protein fragment into the vector, in such a manner that the second test protein is capable of being expressed as part of a hybrid protein with the transcriptional activation domain.

14. (Amended) A method according to [any preceding] claim 9, wherein the $\sigma 54$ transcriptional activator is selected from the group consisting of:

dbj | BAA16379.1 | (D90877) FORMATE HYDROGENLYASE TRANSCRIPTIONAL ACTIVATOR;

emb | CAA26472.1 | (X02616) pot. Nifa gene product (aa 1-484) [Klebsiella pneumoniae];

emb | CAA53584.1 | (X75972) anfa [Rhodobacter capsulatus];

emb | CAA92413.1 | (Z68203) nifa homologue [Rhizobium sp.];

emb | CAA93242.1 | (Z69251) mopr [Acinetobacter calcoaceticus];

emb | CAB53157.1 | (X07567) nifal [Rhodobacter capsulatus];

emb | CAB56537.1 | (AJ249642) response regulator [Pseudomonas stutzeri];

gb | AAA58220.1 | (U18997) ORF_o532 [Escherichia coli];

gb | AAA99303.1 | (L43064) regulatory protein [Pseudomonas aeruginosa];

gb | AAB91397.1 | (AF033203) nifaii protein [Rhodobacter capsulatus];

gb | AAC05586.1 | (AF006075) regulatory protein [Bacillus subtilis];

gb | AAC37124.1 | (L81176) fleq [Pseudomonas aeruginosa];

gb | AAC45640.1 | (AF010585) putative sigma 54 activator [Caulobacter crescentus];

gb | AAC46367.1 | (AF014113) two-component response regulator [Vibrio cholerae];

gb | AAD34591.1 | AF145956_1 (AF145956) transcriptional activator nifa [Rhodospirillum rubrum];

gb | AAD38416.1 | (AF155934) nifa [Alcaligenes faecalis];

gb | AAF28395.1 | (AF069392) flam [Vibrio parahaemolyticus];

gb | AAF33506.1 | (AF170176) Salmonella typhimurium transcriptional regulatory protein;

gb | AAF61932.1 | (AF230804) sigma-54 activator protein ActI [Myxococcus xanthus];

gb | AAF85342.1 | AE004061_7 (AE004061) two-component system, regulatory protein [Xylella fastidiosa];

gb | AAF94676.1 | (AE004230) sigma-54 dependent response regulator [Vibrio cholerae];

gb | AAF95280.1 | (AE004286) sigma-54 dependent response regulator [Vibrio cholerae];

gb | AAF96095.1 | (AE004358) sigma-54 dependent transcriptional regulator [Vibrio cholerae];

gb | AAG01527.1 | AF288483_1 (AF288483) nifa [Azospirillum brasilense];

15. (Amended) A method according to [any one of] claim[s 1 to] 14, wherein the $\sigma 54$ transcriptional activator is the NifA transcriptional activator or the PspF transcriptional activator.
16. (Amended) A method according to [any one of] claim[s 1 to] 14, wherein the hybrid $\sigma 54$ transcriptional activator is NifA and activation resulting from NifA- $\sigma 54$ RNAP interaction is enhanced by the coexpression of wild-type or mutant NifA.
17. A method according to claim 16, wherein the hybrid $\sigma 54$ transcriptional activator is NifA from *Azotobacter vinelandii*, and the wild-type or mutant NifA is NifA from *Klebsiella pneumoniae*.
18. (Amended) A method according to [any preceding] claim 9, wherein the nucleic acid molecule comprises a binding site for a factor which induces DNA bending.
19. A method according to claim 18, wherein the factor is integration host factor (IHF).
20. (Amended) A method according to [any one of] claim[s 1 to] 17, wherein the nucleic acid molecule comprises DNA that is intrinsically bent.
21. (Amended) A method according to [any preceding] claim 20, wherein the nucleic acid molecule comprises a nifH promoter from *A. vinelandii* driving a reporter gene.
22. (Amended) A method according to [any preceding] claim 21, wherein the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (lacZ, lacY and lacA); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.
23. (Amended) A method according to [any preceding] claim 22, which is carried out in the presence of a compound which modifies protein-protein or protein-DNA interaction.

24. A method according to claim 22, wherein the compound is selected from the group consisting of molecules which alter the structure of the DNA-binding protein; molecules which alter the structure of DNA; and molecules which modify protein-protein interactions.
25. (Amended) A method according to [any preceding] claim 24, which is carried out *in vivo*.
26. A method according to claim 25, wherein the *in vivo* host is *E.coli*.
27. (Amended) A method according to [any one of] claim[s 1 to] 24, which is carried out *in vitro*.

APPENDIX 2

1. A method for detecting a protein-nucleic acid interaction between a nucleic acid molecule and a protein molecule, comprising the steps of:

a) providing one or more hybrid $\sigma 54$ activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active $\sigma 54$ transcription activating domain;

b) providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for $\sigma 54$ RNA polymerase (RNAP), which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain; and

c) detecting expression of the reporter gene.

2. A method according to claim 1, comprising providing a repertoire of hybrid $\sigma 54$ activator proteins, said repertoire comprising a plurality of different nucleic acid binding sequences.

3. A method according to claim 1, comprising providing a repertoire of hybrid nucleic acid molecules, said repertoire comprising a plurality of different binding sites for the nucleic acid binding sequence.

4. A method according to claim 1, comprising providing both a repertoire according to claim 2 and a repertoire according to claim 3.

5. A method for detecting a protein-protein interaction, comprising the steps of:

a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;

b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active $\sigma 54$ transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a hybrid protein comprising both a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain; and

e) detecting expression of the reporter gene.

6. A method according to claim 5, comprising providing a repertoire of first hybrid proteins, said repertoire comprising a plurality of bait polypeptides.

7. A method according to claim 5, comprising providing a repertoire of second hybrid proteins, said repertoire comprising a plurality of prey polypeptides.

8. A method according to claim 5, comprising providing a repertoire of first hybrid proteins and a repertoire of second hybrid proteins, said repertoires comprising a plurality of bait and prey polypeptides.

9. A method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

b) providing a $\sigma 54$ activator protein comprising a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain;

14. (Amended) A method according to claim 9, wherein the σ 54 transcriptional activator is selected from the group consisting of:

dbj | BAA16379.1 | (D90877) FORMATE HYDROGENLYASE TRANSCRIPTIONAL ACTIVATOR;
emb | CAA26472.1 | (X02616) pot. Nifa gene product (aa 1-484) [Klebsiella pneumoniae];
emb | CAA53584.1 | (X75972) anfa [Rhodobacter capsulatus];
emb | CAA92413.1 | (Z68203) nifa homologue [Rhizobium sp.];
emb | CAA93242.1 | (Z69251) mopr [Acinetobacter calcoaceticus];
emb | CAB53157.1 | (X07567) nifal [Rhodobacter capsulatus];
emb | CAB56537.1 | (AJ249642) response regulator [Pseudomonas stutzeri];
gb | AAA58220.1 | (U18997) ORF_o532 [Escherichia coli];
gb | AAA99303.1 | (L43064) regulatory protein [Pseudomonas aeruginosa];
gb | AAB91397.1 | (AF033203) nifaii protein [Rhodobacter capsulatus];
gb | AAC05586.1 | (AF006075) regulatory protein [Bacillus subtilis];
gb | AAC37124.1 | (L81176) fleq [Pseudomonas aeruginosa];
gb | AAC45640.1 | (AF010585) putative sigma 54 activator [Caulobacter crescentus];
gb | AAC46367.1 | (AF014113) two-component response regulator [Vibrio cholerae];
gb | AAD34591.1 | AF145956_1 (AF145956) transcriptional activator nifa [Rhodospirillum rubrum];
gb | AAD38416.1 | (AF155934) nifa [Alcaligenes faecalis];
gb | AAF28395.1 | (AF069392) flam [Vibrio parahaemolyticus];
gb | AAF33506.1 | (AF170176) Salmonella typhimurium transcriptional regulatory protein;
gb | AAF61932.1 | (AF230804) sigma-54 activator protein ActI [Myxococcus xanthus];
gb | AAF85342.1 | AE004061_7 (AE004061) two-component system, regulatory protein [Xylella fastidiosa];
gb | AAF94676.1 | (AE004230) sigma-54 dependent response regulator [Vibrio cholerae];
gb | AAF95280.1 | (AE004286) sigma-54 dependent response regulator [Vibrio cholerae];
gb | AAF96095.1 | (AE004358) sigma-54 dependent transcriptional regulator [Vibrio cholerae];
gb | AAG01527.1 | AF288483_1 (AF288483) nifa [Azospirillum brasilense];

pir || A48291 ornithine decarboxylase inhibitor - Eschenchia coil;
 pir || B49940 nitrogen regulator I homolog - Escherichia coil;
 pir || C70320 transcription regulator nifa family - Aquifex aeolicus;
 pir || C70396 transcription regulator ntrc family - Aquifex aeolicus;
 pir || C70454 transcription regulator ntrc family - Aquifex aeolicus;
 pir || D70315 transcription regulator ntrc family - Aquifex aeolicus;
 pir || H69581 transcription activator of acetoin dehydrogenase operon acor – Bacillus subtilis;
 pir || I39719 nitrogen regulatory protein - Agrobacterium tumefaciens;
 pir || JC5471 regulatory protein nifa - Azospirillum lipoferum;
 pir || T08624 probable ntrc-type response regulator - Eubacterium acidaminophilum;
 sp | P03027 | NIFA_KLEPN NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P09570 | NIFA_AZOVI NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P12627 | VNFA_AZOVI NITROGEN FIXATION PROTEIN VNFA;
 sp | P14375 | HYDG_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
 sp | P21712 | YFHA_ECOLI HYPOTHETICAL 49.1 KD PROTEIN IN GLNB-PURL
 INTERGENIC REGION (ORFXB);
 sp | P24426 | NIFA_RHLT NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P25852 | HYDG_SALTY TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
 sp | P27713 | NIFA_HERSE NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P30667 | NIFA_AZOBR NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P38035 | RTCR_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN RTCR;
 sp | P54929 | NIFA_AZOLI NIF-SPECIFIC REGULATORY PROTEIN;
 sp | P56266 | NIFA_KLEOX NIF-SPECIFIC REGULATORY PROTEIN;
 sp | Q06065 | ATOC_ECOLI ACETOACETATE METABOLISM REGULATORY
 PROTEIN ATOC (ORNITHINE/ARGININE);
 sp | Q46802 | YGEV_ECOLI HYPOTHETICAL SIGMA-54-DEPENDENT
 TRANSCRIPTIONAL REGULATOR IN;
 sp | Q53206 | NIFA_RHISN NIF-SPECIFIC REGULATORY PROTEIN; and
 sp | Q9ZIB7 | TYRR_ERWHE TRANSCRIPTIONAL REGULATORY PROTEIN TYRR.

15. (Amended) A method according to claim 14, wherein the $\sigma 54$ transcriptional activator is the NifA transcriptional activator or the PspF transcriptional activator.
16. (Amended) A method according to claim 14, wherein the hybrid $\sigma 54$ transcriptional activator is NifA and activation resulting from NifA- $\sigma 54$ RNAP interaction is enhanced by the coexpression of wild-type or mutant NifA.
17. A method according to claim 16, wherein the hybrid $\sigma 54$ transcriptional activator is NifA from *Azotobacter vinelandii*, and the wild-type or mutant NifA is NifA from *Klebsiella pneumoniae*.
18. (Amended) A method according to claim 9, wherein the nucleic acid molecule comprises a binding site for a factor which induces DNA bending.
19. A method according to claim 18, wherein the factor is integration host factor (IHF).
20. (Amended) A method according to claim 17, wherein the nucleic acid molecule comprises DNA that is intrinsically bent.
21. (Amended) A method according to claim 20, wherein the nucleic acid molecule comprises a nifH promoter from *A. vinelandii* driving a reporter gene.
22. (Amended) A method according to claim 21, wherein the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (lacZ, lacY and lacA); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.
23. (Amended) A method according to claim 22, which is carried out in the presence of a compound which modifies protein-protein or protein-DNA interaction.

24. A method according to claim 22, wherein the compound is selected from the group consisting of molecules which alter the structure of the DNA-binding protein; molecules which alter the structure of DNA; and molecules which modify protein-protein interactions.
25. (Amended) A method according to claim 24, which is carried out *in vivo*.
26. A method according to claim 25, wherein the *in vivo* host is *E coli*.
27. (Amended) A method according to claim 24, which is carried out *in vitro*.

Selection System

The present invention relates to a screening system useful for screening repertoires of DNA binding domains. In particular the invention relates to a screening system based on transcriptional activators of bacterial $\sigma 54$ -dependent promoters.

The majority of proteins involved in cellular functions do so by interacting with other proteins or nucleic acid sequences within the cell. Several approaches have been described that allow the *in vivo* selection of nucleic acids which express polypeptides capable of binding to proteins or DNA in the cell. Arguably the most powerful approaches are the yeast one- and two hybrid systems (Fields S. & Song O. (1989) *Nature* 340, 245; see US Patent 5,283,173, incorporated herein by reference in its entirety) for the screening of protein-DNA and protein-protein interactions, respectively. However, the two-hybrid system requires an eukaryotic host and consequently the diversity that can be screened is limited. Furthermore the system notoriously suffers from an abundance of false positives.

Larger molecular repertoires can be prepared in bacterial hosts and a number of bacterial systems for the screening of protein-protein and protein-DNA interactions have also been reported. Two systems have been put forward in which the polypeptide chain of an enzyme is expressed in two parts fused to two candidate polypeptides, and in which interaction between the candidate polypeptides reconstitutes the function of the enzyme (Karimova G. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 5752; Pelletier J.N. et al (1998) *Proc. Nat. Acad. Sci USA* 95, 12141).

Several *in vivo* screens for DNA-binding proteins have also been reported (reviewed in Mossing M.C., Bowie J.U. & Sauer R.T. (1991) *Methods Enzymol.* 208, 604; Elledge S.J. et al (1989) *Proc. Nat. Acad. Sci USA* 86, 3689). Each of these methods involves the blockage of a hybrid $\sigma 70$ promoter by the DNA binding protein. Repression of the promoter either prevents the production of conditionally toxic gene or alleviates repression of an antibiotic gene by transcriptional interference. The transcriptional interference assay (Elledge *et al.*) has been used successfully in one case to select DNA binding proteins with altered specificity (Sera T. & Schultz P.G. (1996) *Proc. Nat. Acad. Sci USA* 93, 2920).

Another $\sigma 70$ -based system utilises recruitment of the polymerase to the promoter by way of a protein-protein interaction between a protein domain fused to the RNA polymerase α subunit and another fused to the lambda repressor bound immediately upstream of the RNA polymerase promoter binding site (Dove S.L., Joung J.K., Hochschild A. (1997),
5 *Nature* 386, 627). By replacing the lambda repressor DNA binding domain with a library of Zn-finger domains, specific DNA binding Zn-finger domains were selected (Joung J.K., Ramm E.I., Pabo C.O. (2000) *Proc Natl Acad Sci U S A*, 97, 7382)

The alternative holoenzyme form of bacterial RNA polymerase (RNAP) contains the $\sigma 54$ factor ($\sigma 54$ -RNAP). As has been previously shown, this polymerase, in most cases, forms
10 a closed complex with the promoter. Unlike $\sigma 70$ promoters at which the RNA polymerase is bound in an active form and is largely controlled by repression, the $\sigma 54$ RNA polymerase holoenzyme is transcriptionally incompetent and is unable to initiate transcription by itself. Initiation of transcription requires the presence of a transcriptional
15 activator that catalyses the isomerisation of the closed promoter complex to an open one. Typically, activator proteins bind to a specific upstream activation sequence (UAS) located 80 to 200 bp upstream of the $\sigma 54$ core promoter. The function of the UAS is to tether the activator in the right position and to bring it in the vicinity of the promoter in order to increase the efficiency of interaction between the $\sigma 54$ RNAP and the activator.
20 Transcriptional activators of $\sigma 54$ dependent promoters have been called bacterial enhancers because their mechanism of activation is superficially similar to the activation of transcription by enhancer proteins in eukaryotes (Kustu S. et al (1991) *Trends Biochem Sci* 16, 397).

25 Conversion of the $\sigma 54$ RNAP into an active form is catalysed by the binding of an enhancer protein coupled to hydrolysis of ATP. This unusual mechanism accounts for the low level of background transcription and the enormous difference (10^4 - 10^5) between on and off states in the strongest $\sigma 54$ promoters effected by a single factor. In comparison, activators of $\sigma 70$ promoters such as CAP or λ cI increase transcription levels usually by
30 less than 10-fold.

- Transcriptional activators of $\sigma 54$ promoters (also known as enhancer-binding proteins or EBPs) share a common structure (see Morrett and Segovia, (1993) J. Bacteriol. 6067-6074) comprising a non-conserved N-terminal domain which has a putative regulatory function, a central domain which is responsible for transcriptional activation, and a C-terminal DNA binding domain which binds the relevant UAS in the target gene. The domains are modular: the central and N-terminal domains together are capable of constitutive activation of $\sigma 54$ RNAP when overexpressed. At least in some cases, the isolated DNA binding domain is capable of specifically binding its DNA recognition site.
- In many instances, interaction between $\sigma 54$ RNAP and the activator is enhanced by a cellular factor which promotes DNA bending between the UAS and the $\sigma 54$ promoter (Freundlich *et al.*, (1992) Mol. Microbiol. 6:2557-2563). This factor, known as integration host factor (IHF) acts to promote transcription from $\sigma 54$ promoters.

Summary of the Invention

We provide herein a novel screening system which is based on transcriptional activators of $\sigma 54$ -based promoters.

- According to a first aspect of the invention, therefore, there is provided a method for detecting a protein-nucleic acid interaction between a nucleic acid molecule and a protein molecule, comprising the steps of:
- providing one or more hybrid $\sigma 54$ activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active $\sigma 54$ transcription activating domain;
 - providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for $\sigma 54$ RNAP, which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain; and
 - detecting expression of the reporter gene.

The invention provides a reporter system which is characterised by very low levels of background expression, since the $\sigma 54$ polymerase is transcriptionally incompetent in the absence of a $\sigma 54$ transcriptional activator. Since, at physiological concentrations, the binding of the transcriptional activator to the nucleic acid is required in order to activate transcription by $\sigma 54$ RNAP, the system of the invention may be used as a tool for investigating and/or screening protein/nucleic acid interactions exploiting the reporter gene read-out.

In the first aspect of the invention, either the nucleic acid binding protein or the nucleic acid molecule may be provided in the form of a repertoire of molecules. Repertoires of hybrid $\sigma 54$ activator proteins preferably are partially or completely randomised at least in the heterologous nucleic acid binding sequence. This allows selection from the library of molecules having desired nucleic acid binding characteristics.

Repertoires of nucleic acid molecules advantageously are partially or completely randomised in the binding site for the nucleic acid binding sequence of the $\sigma 54$ activator protein. This allows selection of nucleic acid molecules having desired binding sites for the chimeric activators.

In a second aspect of the invention, there is provided a system for selecting protein-protein interactions based on the constitutively active hybrid $\sigma 54$ activators described above. The system according to the invention is conceptually similar to the yeast two-hybrid system.

Accordingly, there is provided a method for detecting a protein-protein interaction, comprising the steps of:

- a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;
- b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active $\sigma 54$ transcription activating domain;
- c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a

reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain:

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a
5 hybrid protein comprising both a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain: and

e) detecting expression of the reporter gene.

As will be apparent to those skilled in the art, reference to a "binding site" for the nucleic
10 acid binding sequence includes the provision of several appropriately spaced binding sites in the nucleic acid molecule.

As with the yeast two-hybrid system, in which a modular transcription factor is assembled through binding of DNA binding domain/bait and transcription activating domain/prey
15 hybrids, the association of the nucleic acid binding sequence and the $\sigma 54$ transcription activating domain through the bait/prey interaction allows the detection of, and screening for, protein-protein binding interactions *in vivo* and *in vitro*. Advantageously, the bait and/or prey polypeptide sequences are provided in the form of repertoires, which may be partially or completely randomised. This allows selection of prey polypeptides based on
20 their ability to form interactions with a desired bait (or vice versa). As the assay may be conducted *in vivo*, in a bacterium, the invention permits the detection of *in vivo* binding interactions between polypeptides in bacteria.

It will be apparent that the hybrid proteins useful in the methods of the invention are
25 advantageously provided in the form of nucleic acid vectors or libraries thereof capable of expressing said proteins in a host bacterium. Advantageously, the vector(s) include first and second chimeric genes which encode the hybrid proteins of the invention. Preferably, the vectors also include means for replication in bacteria. Also included may be one or more marker genes, the expression of which in the bacterium permits selection of cells
30 containing the vector(s) from cells that do not contain the vector(s). Preferably, the vector(s) are plasmid(s).

In a third aspect, the invention provides a method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

5 b) providing a $\sigma 54$ activator protein comprising a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain;

10 c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the repertoire and $\sigma 54$ activator together with the nucleic acid molecule in a HIF⁺ host cell, such that $\sigma 54$ activator and the nucleic acid molecule may interact, and transcription activated from the $\sigma 54$ RNAP binding site in a manner dependent on DNA bending by the polypeptide factors; and

15 e) detecting expression of the reporter gene.

It is known that activation by $\sigma 54$ activators may be regulated by factors which induce DNA bending in the target gene. For example, the host factor IHF is known to potentiate $\sigma 54$ activation; moreover, it may be replaced by alternative DNA bending polypeptides, or
20 by intrinsically bent DNA.

The invention moreover provides methods for development of improved $\sigma 54$ activator-based tools.

25 The first chimeric gene includes a nucleic acid sequence that encodes a nucleic-binding domain and a first (bait) test protein or protein fragment in such a manner that the first test protein is expressed as part of a hybrid protein with the nucleic acid-binding domain.

The second chimeric gene also includes a promoter and a transcription termination signal
30 to direct transcription. The second chimeric gene moreover includes a nucleic acid sequence that encodes a $\sigma 54$ transcriptional activation domain and a second (prey) test protein or protein fragment into the vector, in such a manner that the second test protein is

capable of being expressed as part of a hybrid protein with the transcriptional activation domain.

The invention moreover provides kits for practising the invention, which kits
5 advantageously comprise a container, two vectors, and a host cell. The first vector contains a promoter and may include a transcription termination signal functionally associated with the first chimeric gene in order to direct the transcription of the first chimeric gene. The chimeric gene advantageously comprises one or more unique restriction site(s) to insert a nucleic acid sequence encoding a test bait polypeptide. The kit also may also include a
10 second vector which contains a second chimeric gene, optionally comprising one or more unique restriction site(s) to insert a nucleic acid sequence encoding the prey polypeptide; alternatively, the second chimeric gene may be present on the same vector as the first chimeric gene.

15 Brief description of the Figures

Figure 1A is a schematic representation of $\sigma 54$ RNAP activation by $\sigma 54$ activator NifA.

Figure 1B is a schematic representation of the invention, in which the $\sigma 54$ DNA binding
20 domain is replaced with a heterologous GCN4 DNA binding domain..

Figure 2 is a schematic representation of the first aspect of the present invention, in which a library of DNA binding domains is screened together with a library of DNA binding domain binding sites to identify protein:DNA binding pairs.
25

Figure 3 shows the activation of transcription by NifA-chimera as expressed as percent of wt activity (NifA/UAS). Nif-GCN4 (in presence of the NifA Δ C coactivator (NifADC)) show close to wt activity. Equal activity is observed for the two distinct GCN4 DNA recognition sites (ATF/Creb and AP-1). Less than 1% wt activity is observed with a non-cognate reporter such as one bearing the wt nifH UAS.
30

Figure 4 shows the activation of transcription by NifA-chimera as expressed as percent of wt activity (NifA/UAS). Nif-ERDBD (in presence of the NifAΔC coactivator (NifADC)) shows ca. 80% of wt activity. Very little activation is observed with a non-cognate reporter bearing the DNA recognition site (GRE) for the closely related Glucocorticoid receptor.

Figure 5 shows the coactivation by different NifA variants as expressed as percent of wt activity (NifAwt). NifA from *Klebsiella pneumoniae* (NifAKp) is superior to all others, even exceeding wt activity (up to 160%). NifAKp with its DNA domain deleted (NifAΔCKp (NifADCKp)) is almost as active.

Detailed Description of the Invention

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in bacterial cell culture, molecular genetics, nucleic acid chemistry, protein chemistry and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed. John Wiley & Sons, Inc. which are incorporated herein by reference), chemical methods, pharmaceutical formulations and delivery and treatment of patients.

A: Nucleic Acids and Proteins

As used herein, "nucleic acid" refers to any natural nucleic acid, including RNA and DNA as well as synthetic nucleic acid comprising modified or synthetic bases, and mixtures of modified or synthetic bases with natural bases. Such modified and/or synthetic bases may be referred to as derivatives of DNA or RNA. Preferably, "nucleic acid" refers to DNA.

The invention includes the use of modified and/or artificial "nucleic acids". A number of modifications have been described that alter the chemistry of the phosphodiester backbone, sugars or heterocyclic base components of nucleic acids.

5 Among useful changes in the backbone chemistry are phosphorothioates; phosphorodithioates, where both of the non-bridging oxygens are substituted with sulphur; phosphoroamidites; alkyl phosphotriesters and boranophosphates. Achiral phosphate derivatives include 3'-O'-5'-S-phosphorothioate, 3'-S-5'-O-phosphorothioate, 3'-CH₂-5'-O-phosphonate and 3'-NH-5'-O-phosphoroamidate. Peptide nucleic acids
10 replace the entire phosphodiester backbone with a peptide linkage.

Sugar modifications are also known. The α -anomer of deoxyribose may be used, where the base is inverted with respect to the natural β -anomer. The 2'-OH of the ribose sugar may be altered to form 2'-O-methyl or 2'-O-allyl sugars, which provides resistance to
15 degradation without comprising affinity.

Modification of the heterocyclic bases must maintain proper base pairing. Some useful substitutions include deoxyuridine for deoxythymidine; 5-methyl-2'-deoxycytidine and 5-bromo-2'-deoxycytidine for deoxycytidine. 5-propynyl-2'-deoxyuridine and
20 5-propynyl-2'-deoxycytidine have been shown maintain biological activity when substituted for deoxythymidine and deoxycytidine, respectively.

As used herein, the term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by
25 covalent or non-covalent means. As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulphide bonds. The term domain also refers to polypeptides and peptides having biological function. A peptide useful in the invention will have a binding or transcription activating capability, i.e., with respect to binding to nucleic acids, other
30 proteins or polypeptides, and activation of σ 54 RNAP transcription. It also may have another biological function that is a biological function of a protein or domain from which the peptide sequence is derived.

A hybrid protein is a protein or polypeptide which comprises constituent parts derived from at least two naturally-occurring or artificial proteins. In particular, it may comprise the DNA-binding domain of one protein and the protein-binding or transcription activating domain of a second protein.

B: $\sigma 54$ Activators

Activators of $\sigma 54$ transcription are well known and have been reviewed, for example, by Buck *et al.*, J Bacteriol. 2000 Aug;182(15):4129-36; Studholme and Buck, FEMS Microbiol Lett. 2000 May 1;186(1):1-9; Shingler, Mol Microbiol. 1996 Feb;19(3):409-16; Goosen and van der Putte, Mol Microbiol. 1995 Apr;16(1):1-7; Merrick, Mol Microbiol. 1993 Dec;10(5):903-9; and others. A family of such activator proteins has been defined, and its members found to share homology in the central (catalytic) domain which is responsible for $\sigma 54$ RNAP activation.

Members of the family include the following (the numbers are GenBank accession numbers)

- 20 dbj|BAA16379.1| (D90877) FORMATE HYDROGENLYASE TRANSCRIPTIONAL ACTIVATOR.
- emb|CAA26472.1| (X02616) pot. NifA gene product (aa 1-484) [Klebsiella pneumoniae]
- emb|CAA53584.1| (X75972) anfA [Rhodobacter capsulatus]
- emb|CAA92413.1| (Z68203) NifA homologue [Rhizobium sp.]
- 25 emb|CAA93242.1| (Z69251) MopR [Acinetobacter calcoaceticus]
- emb|CAB53157.1| (X07567) NifA1 [Rhodobacter capsulatus]
- emb|CAB56537.1| (AJ249642) response regulator [Pseudomonas stutzeri]
- gb|AAA58220.1| (U18997) ORF_o532 [Escherichia coli]
- gb|AAA99303.1| (L43064) regulatory protein [Pseudomonas aeruginosa]
- 30 gb|AAB91397.1| (AF033203) NifAII protein [Rhodobacter capsulatus]
- gb|AAC05586.1| (AF006075) regulatory protein [Bacillus subtilis]
- gb|AAC37124.1| (L81176) FleQ [Pseudomonas aeruginosa]

- gb|AAC45640.1| (AF010585) putative sigma 54 activator [*Caulobacter crescentus*]
 gb|AAC46367.1| (AF014113) two-component response regulator [*Vibrio cholerae*]
 gb|AAD34591.1|AF145956_1 (AF145956) transcriptional activator NifA
 [Rhodospirillum rubrum]
- 5 gb|AAD38416.1| (AF155934) NifA [*Alcaligenes faecalis*]
 gb|AAF28395.1| (AF069392) FlaM [*Vibrio parahaemolyticus*]
 gb|AAF33506.1| (AF170176) Salmonella typhimurium transcriptional regulatory protein
 gb|AAF61932.1| (AF230804) sigma-54 activator protein Act1 [*Myxococcus xanthus*]
 gb|AAF85342.1|AE004061_7 (AE004061) two-component system. regulatory protein
- 10 [Xylella fastidiosa]
 gb|AAF94676.1| (AE004230) sigma-54 dependent response regulator [*Vibrio cholerae*]
 gb|AAF95280.1| (AE004286) sigma-54 dependent response regulator [*Vibrio cholerae*]
 gb|AAF96095.1| (AE004358) sigma-54 dependent transcriptional regulator [*Vibrio cholerae*]
- 15 gb|AAG01527.1|AF288483_1 (AF288483) NifA [*Azospirillum brasilense*]
 pir|A48291 ornithine decarboxylase inhibitor - *Escherichia coli*
 pir|B49940 nitrogen regulator I homolog - *Escherichia coli*
 pir|C70320 transcription regulator NifA family - *Aquifex aeolicus*
 pir|C70396 transcription regulator NtrC family - *Aquifex aeolicus*
- 20 pir|C70454 transcription regulator NtrC family - *Aquifex aeolicus*
 pir|D70315 transcription regulator NtrC family - *Aquifex aeolicus*
 pir|H69581 transcription activator of acetoin dehydrogenase operon acoR - *Bacillus subtilis*
 pir|I39719 nitrogen regulatory protein - *Agrobacterium tumefaciens*
- 25 pir|JC5471 regulatory protein NifA - *Azospirillum lipoferum*
 pir|T08624 probable NtrC-type response regulator - *Eubacterium acidaminophilum*
 sp|P03027|NIFA_KLEPN NIF-SPECIFIC REGULATORY PROTEIN
 sp|P09570|NIFA_AZOVI NIF-SPECIFIC REGULATORY PROTEIN
 sp|P12627|VNFA_AZOVI NITROGEN FIXATION PROTEIN VNFA
- 30 sp|P14375|HYDG_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN HYDG
 sp|P21712|YFHA_ECOLI HYPOTHETICAL 49.1 KD PROTEIN IN GLNB-PURL
 INTERGENIC REGION (ORFXB)

- sp|P24426|NIFA_RHILT NIF-SPECIFIC REGULATORY PROTEIN
 sp|P25852|HYDG_SALTY TRANSCRIPTIONAL REGULATORY PROTEIN HYDG
 sp|P27713|NIFA_HERSE NIF-SPECIFIC REGULATORY PROTEIN
 sp|P30667|NIFA_AZOB R NIF-SPECIFIC REGULATORY PROTEIN
 5 sp|P38035|RTCR_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN RTCR
 sp|P54929|NIFA_AZOLI NIF-SPECIFIC REGULATORY PROTEIN
 sp|P56266|NIFA_KLEOX NIF-SPECIFIC REGULATORY PROTEIN
 sp|Q06065|ATOC_ECOLI ACETOACETATE METABOLISM REGULATORY
 PROTEIN ATOC (ORNITHINE/ARGININE
 10 sp|Q46802|YGEV_ECOLI HYPOTHETICAL SIGMA-54-DEPENDENT
 TRANSCRIPTIONAL REGULATOR IN
 sp|Q53206|NIFA_RHISN NIF-SPECIFIC REGULATORY PROTEIN
 sp|Q9ZIB7|TYRR_ERWHE TRANSCRIPTIONAL REGULATORY PROTEIN TYRR
- 15 Moreover, a number of polypeptides belonging to the σ 54 activator family have been
 described whose 3D structures are known. These include: 113161 acetoin catabolism
 regulatory protein; 113629 alginate biosynthesis transcriptional regulatory protein ALGB;
 266789 type 4 fimbriae expression regulatory protein PILR; 113833 nitrogen fixation
 protein ANFA; 138884 nitrogen fixation protein VNFA; 128219 nif-specific regulatory
 20 protein; 3024194 nif-specific regulatory protein; acetoacetate metabolism regulatory
 protein ATOC 1168553 (ornithine/arginine decarboxylase inhibitor) (ornithine
 decarboxylase antizyme); 417166 transcriptional regulatory protein HYGD; 266622 nif-
 specific regulatory protein; 1352500 nif-specific regulatory protein; 128224 nif-specific
 regulatory protein; 128225 nif-specific regulatory protein; 128221 nif-specific regulatory
 25 protein; 128226 nif-specific regulatory protein; 1346014 transcriptional regulatory protein
 FLBD; 549560 hypothetical sigma-54-dependent transcriptional regulator in GUTQ-HYPF
 intergenic region; 139857 transcriptional regulatory protein XYLR (67 kd protein); 120053
 formate hydrogenlyase transcriptional activator; 2507375 hypothetical 49.1 kd protein in
 GLNB-PUR1 intergenic region (ORFXB) (orf-2); 134961 signal-transduction and
 30 transcriptional-control protein; 1171795 nitrogen assimilation regulatory protein; 417388
 nitrogen regulation protein nr(i); 123466 hydrogenase transcriptional regulatory protein
 HOXA; 399925 hydrogenase transcriptional regulatory protein HOXA; 585586 nitrogen

assimilation regulatory protein NTRX; 118399 c4-dicarboxylate transport transcriptional regulatory protein DCTD; 585267 pathogenicity locus probable regulatory protein HRPR; 1346313 pathogenicity locus probable regulatory protein HRPS; 549447 pathogenicity locus probable regulatory protein WTSA; 585909 arginine utilization regulatory protein ROCR; 136600 transcriptional regulatory protein TYRR; 1174836 transcriptional regulatory protein TYRR homolog; 123748 hydrogenase transcriptional regulatory protein HUPR1; 128604 nitrogen regulation protein NTRC; 1169293 glycerol metabolism operon regulatory protein; and 129957 phosphoglycerate transport system transcriptional regulatory protein PGTA . The numbers are GenBank gi numbers.

10

Preferably, the hybrid $\sigma 54$ activator is based on the NifA activator. The Nif family of bacterial enhancers regulate expression of nitrogenase components from $\sigma 54$ promoters in nitrogen-fixing bacteria, and are inhibited by NifL (Austin S. et al (1994) *J. Bacteriol.* 176, 3460). In bacteria lacking NifL, NifA is constitutively active. NifA is modular in architecture and it is shown herein that this allows for the swapping of the natural DNA-binding domain (DBD) for heterologous DBDs. Such NifA-DBD chimaeras are inactive on the wild type promoter, but activate transcription from hybrid promoters bearing their cognate target sequences.

15

Advantageously, the hybrid $\sigma 54$ activator may be based on *E. coli* PspF (see Jovanovic *et al.*, (1996) *J. Bacteriol.* 178:1936-1945). PspF lacks the N-terminal regulatory domain typical of $\sigma 54$ activators, and is constitutively active but negatively regulated by PspA. Thus, in bacteria lacking PspA, PspF is constitutively active.

20

Other $\sigma 54$ activators may be rendered constitutively active by removal of the N-terminal regulatory domain or by appropriate mutation.

25

Nucleic acid binding sequences or domains are known in the art and may be derived from $\sigma 54$ activator proteins or any other DNA binding proteins, whether naturally-occurring or synthetic. Moreover, DNA-binding domains may be synthesised by partial or complete randomisation. Many naturally-occurring DNA-binding proteins contain independently folded domains for the recognition of DNA, and these domains in turn belong to a large

30

number of structural families, such as the leucine zipper, the homeodomain, the "helix-turn-helix", the zinc finger and various other transcription factor families.

C: Libraries

5

The term library refers to a mixture of heterogeneous polypeptides or nucleic acids. The library is composed of members, which have a unique polypeptide or nucleic acid sequence. To this extent, *library* is synonymous with *repertoire*, although in general the term "library" is used herein to denote the source of the repertoire – e.g. a library of nucleic acid molecules which encodes a repertoire of polypeptides. Sequence differences between library members are responsible for the diversity present in the library. The library may take the form of a simple mixture of polypeptides or nucleic acids, or may be in the form organisms or cells, for example bacteria, viruses, animal or plant cells and the like, transformed with a library of nucleic acids. Advantageously, the nucleic acids are incorporated into expression vectors, in order to allow expression of the polypeptides encoded by the nucleic acids. In a preferred aspect, therefore, a library may take the form of a population of host organisms, each organism containing one or more copies of an expression vector containing a single member of the library in nucleic acid form which can be expressed to produce its corresponding polypeptide member. Thus, the population of host organisms has the potential to encode a large repertoire of genetically diverse polypeptide variants.

Libraries of hybrid proteins may be prepared and selected together with libraries of hybrid nucleic acids. "Crossing" of hybrid libraries is performed by combinatorial infection, which has been employed successfully to generate very large antibody libraries (Griffiths et al (1994) EMBO J. 13, 3245).

Although libraries for use in the present invention may be phage libraries, as is known in the art, it is possible to use alternative libraries which are constructed using other vectors, such as plasmids. In any case, the present invention does not require the library to be capable of "display" of the gene product at the bacterial surface, as with phage libraries;

rather, the gene product is preferable expressed intracellularly, and is advantageously not expressed as a fusion with a vector gene product.

DNA binding domain libraries are preferably based on a known DNA binding domain architectures (e.g. basic leucine zipper, bZIP) and may be derived using PCR amplification with "family-specific" primers. Such libraries may be crossed with hybrid-promoters bearing defined target sequences or libraries of target sequences. In addition to providing information on the distribution of members of the family in a given genome, such libraries may be used to identify and study proteins or molecular compounds that modify DNA interaction within a family of DNA binding domains, for example Tax (from HTLV-1) in the case of bZIP proteins.

In an alternative embodiment, they may also be used to select DNA binding domains which conditionally bind their target sequence only in the presence of other factors such as protein cofactors or small molecular compounds, for example drugs that intercalate into DNA or alter the degree of supercoiling or recognise DNA sequences which have been modified chemically (e.g. methylated). The system can also be used "in reverse" i.e. to select proteins or molecular compounds that disrupt a particular DNA-protein interaction or to select DNA binding domains that do not bind a particular target sequence or library thereof.

More advanced libraries are preferably derived directly from genomic DNA or cDNA libraries and selected on hybrid promoters bearing a repertoire of target sequences, comprising either a stretch of randomised sequence or a library of inserts derived from fragmented genomic DNA. Data obtained in this way allows the compilation of a genomic directory of DNA binding domains and the building of a promoter-DNA binding domain interaction map.

D: Hybrid polypeptides

30

The generation of hybrid polypeptides by domain fusion is well known in the art and may be effected by fusing polypeptides or, preferably, by fusing nucleic acids which encode

the polypeptides. It has been known since 1976 that DNA binding and transcriptional activator domains are separable, and can be swapped between proteins: see Ma and Ptashne, who reported (Cell, (1987) 51, 113-119; Cell, (1988) 55, 443-446) that when both the GAL4 N-terminal domain and C-terminal domain are fused together in the same protein, transcriptional activity is induced. Other proteins are also known function as transcriptional activators via the same mechanism. For example, the GCN4 protein of *Saccharomyces cerevisiae* as reported by Hope and Struhl, Cell, 46, 885-894 (1986), the ADR1 protein of *Saccharomyces cerevisiae* as reported by Thukral et al., Molecular and Cellular Biology, 9, 2360-2369, (1989) and the human estrogen receptor, as discussed by Kumar et al., Cell, 51, 941-951 (1987) both contain separable domains for DNA binding and for maximal transcriptional activation.

The same is specifically known of the $\sigma 54$ bacterial transcriptional activators, although a genetic screen based thereon has not been proposed. Therefore, the present invention may be carried out using techniques which are known to those skilled in the art, particularly as applied to 2-hybrid techniques in eukaryotic cells.

Synthesis of chimeric genes for the purposes of the present invention may be carried out by any desired means, including polynucleotide synthesis and mutagenesis approaches. For example, a number of methods for site-directed mutagenesis are known in the art, from methods employing single-stranded phage such as M13 to PCR-based techniques (see "PCR Protocols: A guide to methods and applications", M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (eds.), Academic Press, New York, 1990). Preferably, the commercially available Altered Site II Mutagenesis System (Promega) may be employed, according to the directions given by the manufacturer.

E: Host Cells

Host cells useful in conjunction with the present invention are prokaryotic cells, advantageously bacterial cells. *E. coli* is the preferred host: however, host cells may belong to any species or genus in which $\sigma 54$ RNAP-driven transcription is possible, such as *Klebsiella*, *Rhodobacter*, *Rhizobium*, *Acinetobacter*, *Pseudomonas*, *Escherichia*,

Bacillus, *Caulobacter*, *Vibrio*, *Rhodospirillum*, *Alcaligenes*, *Salmonella*, *Myxococcus*, *Xylella*, *Azospirillum*, *Aquifex*, *Agrobacterium* and other organisms. In *E. coli*, the preferred configuration is a modified strain, in which a truncated form of Nif (or another activator) is coexpressed to boost specific activation (see Methods).

5

Preferably, the host cells lack repressors of the $\sigma 54$ activator being used, such that the transcription activating domain is constitutively active. Repressors may be deleted by genetic mutation and/or selection, or inhibited by expression of antisense constructs, or the like. In general, due to the accessibility of bacterial genetics, especially in *E. coli*,
10 deletion of repressor genes is straightforward to those skilled in the art.

F: Reporter Genes

Reporter genes of various types are known in the art and may be used in conjunction with
15 the present invention. A "reporter gene", as referred to herein, may be the coding sequence which encodes a detectable gene product, or the coding sequence including the necessary control sequences for its expression in accordance with the invention, as appropriate.

20 Advantageously, the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (*lacZ*, *lacY* and *lacA*); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.

25 Certain reporters, such as the LacZ gene, are widely used in bacterial genetics and are useful in the performance of the invention. However, other genes may also be employed, including fluorescent proteins. For example, green fluorescent proteins (GFPs) of cnidarians, which act as their energy-transfer acceptors in bioluminescence, can be used in the invention. A green fluorescent protein, as used herein, is a protein that fluoresces
30 green light, and a blue fluorescent protein is a protein that fluoresces blue light. GFPs have been isolated from the Pacific Northwest jellyfish, *Aequorea victoria*, from the sea pansy, *Renilla reniformis*, and from *Phialidium gregarium*. (Ward et al., 1982,

Photochem. Photobiol. 35: 803-808; Levine et al., 1982, Comp. Biochem. Physiol. 72B: 77-85).

A variety of *Aequorea*-related GFPs having useful excitation and emission spectra have
5 been engineered by modifying the amino acid sequence of a naturally-occurring GFP from
Aequorea victoria. (Prasher et al., 1992, Gene, 111: 229-233; Heim et al., 1994, Proc.
Natl. Acad. Sci. U.S.A., 91: 12501-12504; PCT/US95/14692). As used herein, a
fluorescent protein is an *Aequorea*-related fluorescent protein if any contiguous sequence
10 of 150 amino acids of the fluorescent protein has at least 85% sequence identity with an
amino acid sequence, either contiguous or non-contiguous, from the wild-type *Aequorea*
green fluorescent protein (SwissProt Accession No. P42212). Similarly, the fluorescent
protein may be related to *Renilla* or *Phialidium* wild-type fluorescent proteins using the
same standards.

15 *Aequorea*-related fluorescent proteins include, for example, wild-type (native) *Aequorea*
victoria GFP, whose nucleotide and deduced amino acid sequences are presented in
GenBank Accession Nos. L29345, M62654, M62653 and others *Aequorea*-related
engineered versions of Green Fluorescent Protein, of which some are listed above. Several
of these, i.e., P4, P4-3, W7 and W2 fluoresce at a distinctly shorter wavelength than wild
20 type.

A specific advantage of fluorescent proteins is that they facilitate FACS sorting of cells in
a manner dependent on reporter gene expression (Norman, S.O. (1980). Flow cytometry.
Med. Phys. 7, 609-615; Mackenzie, N.M. & Pinder, A.C. (1986). The application of flow
25 microfluorimetry to biomedical research and diagnosis: a review. *Dev. Biol. Stand.* 64,
181-193).

Other reporter genes may complement auxotrophic mutations, confer antibiotic resistance
or other selectable characteristics to the host bacteria. Reporter genes may be wholly or
30 partly heterologous to the host cell, and introduced by mutagenesis and/or transformation
with appropriate vectors. Alternatively, endogenous σ^{54} -responsive genes may be used
as reporter genes.

The reporter gene also contains a binding site for $\sigma 54$ RNAP. The consensus sequence for $\sigma 54$ RNAP binding is 5' TGGCAC-N5-TTGCa/t 3'. This sequence is located at -12 to -24 with respect to the start of transcription, whilst the more common sigma 70 recognition sequence is situated at -10 to/ -35. Both the GG & GC must be on the same face of the DNA helix.

In order to increase specificity, combinations of two or more reporter genes (preferably in tandem) may be used.

10

Where the reporter gene is chimeric, i.e. comprises heterologous binding sites for the nucleic acid binding sequence and $\sigma 54$ RNAP binding sites incorporated into the same nucleic acid, the spacing between the $\sigma 54$ RNAP binding site and the nucleic acid binding sequence binding site is preferably conserved with respect to the natural gene from which the $\sigma 54$ RNAP binding site is taken. Advantageously, the spacing is at least calculated such that the spatial relationship of the elements on respective faces of the nucleic acid helix is maintained.

Reporter genes advantageously comprise a binding site for a further activation factor, such as IHF. These factors are believed to induce bending of the DNA, thus potentiating activation of $\sigma 54$ RNAP-driven transcription by $\sigma 54$ activators. Alternatively, the DNA itself may be intrinsically bent, thus providing constitutive potentiation of $\sigma 54$ -specific activation.

25 G: Configurations of the Invention

The present invention may be configured in three basic ways: a first configuration, in which reporter gene activation is dependent on the interaction between the nucleic acid and a nucleic acid binding domain on the hybrid protein; a second configuration, in which reporter gene activation is dependent on interaction between bait and prey polypeptides which serves to bring together two or more components of the hybrid protein; and a third configuration, in which reporter gene activation by a $\sigma 54$ activator is dependent on the

30

presence of DNA-bending polypeptides. As referred to herein, an interaction is advantageously a binding interaction.

Where the invention is configured to detect protein-nucleic acid interaction, libraries of proteins and/or nucleic acids may be prepared as described above. Proteins having improved nucleic acid binding, or nucleic acid sequences having improved affinity for protein domains, may be developed by mutagenesis and selection of candidate sequences. Alternatively, protein and/or nucleic acid sequences may be used to identify, *in vivo*, cognate binding partners.

10

Chimeric $\sigma 54$ activators also offer the opportunity to better understand aspects of the process of transcriptional activation at $\sigma 54$ promoters. In the case of NifA, it is known that binding of the target sequence together with ATP binding promotes oligomerisation of NifA. It is believed that it is the oligomer which contacts the polymerase and catalyses the ATP-driven isomerisation of the polymerase holoenzyme. Taking advantage of the superactivation effect described above it may be possible to address questions such as which components of the oligomer (e.g. the DNA-bound NifA vs. NifA Δ C, i.e. NifA with the DNA binding domain removed), which are contacting the polymerase and/or coupling ATP hydrolysis to transcriptional activation etc. Furthermore, usage of NifA Δ C cofactors from different species (together with their diversification by PCR shuffling) allows identification of the sequence regions critical for transcriptional activation and a "maturation" of the NifA Δ C coactivator. Indeed, we have found the NifA from *K. pneumoniae* to be a superior cofactor to *A. vinelandii* NifA. Finally, it may be possible to use chimera of a known DNA binding domains (e.g. GCN4) and a cDNA library as a prokaryotic "enhancer" trap, to isolate $\sigma 54$ activators on a genome-wide scale.

25

Configuration of the invention to detect protein-protein interactions follows the general scheme of the yeast two-hybrid assay, and the reagents used in the invention may be set up accordingly. In general, therefore, the invention will comprise a nucleic acid binding domain-bait fusion, and a prey- $\sigma 54$ activator domain fusion. Although, in general, "bait" refers to a known polypeptide and "prey" to an unknown polypeptide, the terms may be

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used interchangeably. Indeed, the invention comprises configurations in which both bait and prey are known, or both are unknown.

Binding between the bait and the prey result in constitution of a hybrid protein which
 5 comprises both a nucleic acid binding domain and a $\sigma 54$ activator domain. The hybrid protein is able to activate transcription from a reporter gene, thus providing a bait:prey binding-dependent signal.

Protein-protein interactions may be selected using the preferred NifA system, in which the
 10 hybrid $\sigma 54$ transcriptional activator includes the NifA activation domain. The NifA bacterial two-hybrid system may be used for the generation of interaction matrices between cDNA libraries. Ultimately such interaction matrices may yield an interaction map of the proteins of an organism. The invention provides an alternative to the yeast two hybrid system.

15 Systems based on $\sigma 54$ have a number of advantages over the other systems that are available, e.g. the conceptually similar yeast one and two-hybrid system. A bacterial host allows substantially larger repertoires to be obtained and thus a much larger molecular diversity to be screened. In particular, using combinatorial infection, the system of the
 20 invention allows the "crossing" of both $\sigma 54$ -chimera repertoires with libraries of hybrid reporter constructs, thus permitting coevolution of DNA binding domains, and recognition sites, or coselection of DNA binding domains and target sites from genomic libraries.

Because selection in the $\sigma 54$ -based system is based on a positive readout, i.e. activation of
 25 transcription, it is less prone to false positives than other approaches relying on the inhibitory effect of the expressed DNA binding domains, like the transcription interference assay (Elledge S.J. et al (1989) *Proc. Nat. Acad. Sci USA* 86, 3689). In vivo selection in general may result in the selection of novel DNA binding domains that are more attuned to working under realistic conditions, including supercoiling of the recognition site, presence
 30 of a large excess of chromosomal DNA and high protein concentration. Another advantage of the system of the invention is that extremely low levels of the hybrid protein

appear to be sufficient to affect maximum activation of transcription. This is particularly helpful in the case of DNA binding domains that are prone to aggregation.

5 The $\sigma 54$ -based systems of the present invention may be further adapted to take into account potential disadvantages of bacterial expression. For instance, *E. coli* expression may be suboptimal for large eukaryotic transcription factors. However, large eukaryotic proteins can often be split into smaller domains which retain function and are usually readily expressed in *E. coli*.

10 According to the third configuration, a constitutively active $\sigma 54$ activator may be used to screen a library of candidate DNA-bending polypeptides, preferably in a HIF negative host. Since the degree of activation by the $\sigma 54$ activator may be dependent on DNA bending by additional factors, the levels of expression of the reporter gene will be modulated by the DNA-bending activity of the candidate DNA-bending polypeptides.

15 The invention is further described, for the purposes of illustration only, in the following examples.

Examples

20 NifA from *A. vinelandii* is a well-studied member of the family of bacterial enhancers and it is a positive regulator of the expression of nitrogenase components in diazotrophs. It is inhibited by NifL in response to the presence of oxygen or ammonia. When expressed in *E. coli*, which lacks endogenous NifL or an equivalent, NifA is constitutively active. Because
25 of the highly conserved nature of the activation mechanism of $\sigma 54$ RNA polymerase, NifA is a very strong activator of transcription in *E. coli*.

Like other members of the family of bacterial enhancer proteins, NifA is modular in architecture, both structurally and functionally, comprising 3 domains, a N-terminal sensor domain, a central activation domain (AD), and a C-terminal DNA binding domain (DBD).
30 The central activation domain (AD) can activate transcription independent of DNA

binding if overexpressed. Thus the DBDs function appears to be primarily to increase the Activator domain's concentration in the promoter proximity.

We have exploited the modularity of the enhancer structure and swapped the natural NifA DNA binding domain (DBD) for heterologous DBDs and libraries thereof. Here we describe the activity of these NifA-chimeras in the activation of transcription from the σ^{54} dependent promoter *nifH* and hybrids thereof.

Materials & Methods

Media & Reagents

2xTY, MacConkey agar are described elsewhere (Miller J.H. (1972) *Experiments in molecular genetics*, Cold Spring Harbour, NY). Antibiotics were used at the following concentrations: Ampicillin 0.1 mg/ml, Chloramphenicol 10 μ g/ml, Streptomycin 25 μ g/ml. Min-lac medium was essentially M9 medium (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y) supplemented with 1mM MgSO₄, 20 μ M CaCl₂, 2% (w/v) lactose, 2mg/ml casamino acids, 40 μ g/ml L-tryptophan, 5 μ g/ml thiamine and appropriate antibiotics. Min-lacX plates were essentially M9 plates supplemented 2% lactose, appropriate antibiotics and 40 μ g/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside).

Strains

TG1 Δ K was derived from TG1 (Gibson T. J. (1984) *Studies on the Epstein-Barr virus genome*, University of Cambridge) using the genome integration strategy of Haldimann A. *et al.*, (1996) *Proc. Nat. Acad. Sci USA* 93, 14361. Briefly, NifA (*K. pneumoniae*) residues 1-462 was amplified using Pfu polymerase (Stratagene) and primers 1 (5'- GAG TCA CTA ACG CAT ATG ATC CAT AAA TCC GAT TCG GAC -3'), 2 (5'- CGC GGA TCC AAG CGG CCG CTC ATT AGC GAT GGT TGA ACA GAA TCA C -3') cut with NdeI and BamHI and cloned into the genome targeting suicide vector pSK50D-uidA2 (Haldimann. *Op. Cit.*) and transformed into the Pir⁺ host strain BW23473 (Metcalf W.W. *et al* (1994) *Plasmid* 35, 1). Vectors were isolated and transformed into the Pir⁻ strain TG1 harbouring the plasmid pINT-ts (Hasan N. *et al* (1994) *Gene* 150, 51). Chromosomal

integration was induced by a temperature shift to 42°C, which leads to expression of λ integrase from pINT-ts and simultaneously stops its replication. Integrants were identified by Kanamycin resistance and screened for Nif coactivation. Once obtained TG1ΔK was grown routinely without antibiotic selection.

5

Constructs

Chimeric constructs were based on pDB737 (Austin S. et al (1994) *J. Bacteriol.* 176, 3460 Buck M. et al (1986) *Nature* 320, 374) encoding NifA (*A. vinelandii*) under the control of the T7 promoter in the plasmid pT7-7 (Tabor S. & Richardson C.C. (1985) *Proc Natl Acad Sci USA* 82, 1074). Expression was by leakiness of the T7 promoter. Chimeras were constructed taking advantage of a unique BanII cutting site, in the linker region between the central domain of NifA and the DBD. GCN4 was amplified using Pfu polymerase (Stratagene) and primers 3 (5'- GCT GCC AGC GAG AGC CCG CCG CTC GCC GCG ATT GTG CCC GAA TCC AGT GAT CCT -3') and 4 (5'- GAG CTA AAG CTT TTA TTA GCG TTC GCC AAC TAA TTT CTT TAA TCT GGC -3') cut with BanII and Hind3 and ligated into pDB737 cut with BanII and Hind3. ERDBD was amplified using primers 5 (5'- GTC GAC AAC GAG AGC CCG CCG CTC GCC GCG GAA ACG CGT TAC TGC GCT GTT -3') TGC and 6 (5'- GGT CAG CGC GTG GAT CCT TAA CCA CCA CGA CGG TCT TTA CG-3') cut with BanII and BamHI and ligated into pDB737 cut with BanII and BamHI. The vector p737S1 is derived from pDB737 by replacing the bla gene with aadA conferring streptomycin resistance and the insertion of a fl phage origin for packaging of the vector into filamentous phage particles. Briefly, aadA was amplified using primers 7 (5'- TCA GCG CAC GCT GAC GTC GTG GAA ACG GAT GAA GGC ACG AAC -3'), 8 (5'-CCG CCT GGA GGT GGC CAT TAT TTG CCG ACT ACC TTG GTG ATC TCG CC -3') and cut with AatII and MscI and ligated with pDB737 cut with AatII and ScaI. The resulting vector p737S was cut with AatII, ClaI. The fl ori was amplified using primers 9 (5'- GCT GCC GAC TCG ATC GAT GAA TGG CGA ATG GCG CCT GAT GCG G -3'), 10 (5'-CCG GGT CGT GAC GTC AGT GTT GGC GGG TGT CGG GGC TGG C -3') cut with AatII, ClaI and cloned into the cut p737S to give p737S1. NifA-X chimera were transferred from pDB737 to p737S1 by digestion with NdeI . Hind3 (BamHI for NifA-ERDBD).

30

Reporter constructs were derived from pACYC184 and the vector pMB1 (Buck M. *et al.* (1986) *Nature* 320, 374). Briefly the lac-operon (lacZYA) was amplified with primers 11 (5'- GAG TCA ATT CGG GGA TCC CGT CGT TTT ACA ACG TCG TGA CTG G-3'),
12 (5'- GAG TCA TTC TGG CCA GTC GAC CGC TCT GCC GGT GGT TAC -3') and
cut with BamHI and MscI. The nitH promoter segment from pMB1 was amplified with
primers 13 (5'- GAG TCA TTC AAG CTT GCG TGG AAT AAG ACA CAG GGG
GCG-3'), 14 (5'- GAG TCA TTC GGG ATC CCC GGA TTT ACC GAT ACC GCC
TTT ACC -3') and cut with Hind3, BamHI and the 2 fragments simultaneously ligated
with pACYC184 cut with Hind3 and BsaAI to give pMB3. The fl ori was amplified with
primers 15 (5'- GCT GCC GAC TCG GCT AGC GAA TGG CGA ATG GCG CCT GAT
GCG G -3'), 16 (GCC GGG TCG CTT TAA AGT GTT GGC GGG TGT CGG GGC
TGG C -3') and cut with NheI and DraI and ligated into pMB3 cut with both NheI, XmnI
to give pMB31.

Selection and screening

Cells were cotransformed either by simultaneous or sequential electroporation with an
expressor construct and a reporter construct and grown overnight with appropriate
antibiotic selection at 34°C in M9-lac medium and plated out. β -gal expression was scored
either on MacConkey or Minlac-X-gal indicator plates or by ONPG enzyme assay of
selected colonies (see below).

Enzyme assay

ONPG assays used to measure β -gal activity were essentially as described by Kolmar H.
et al. (1995) *EMBO J* 14, 3895. Briefly, 20 μ l of an overnight culture is transferred to a
microtitre well and 100 μ l of chloroform saturated Z-buffer (100mM NaHPO₄, 1mM KCL,
1mM MgSO₄, 50mM β -mercaptoethanol, pH 7.0 (Miller J.H. (1972) *Experiments in
molecular genetics*, Cold Spring Harbour, NY) was added and the optical density at 600nm
determined using an ELISA reader. Cells were lysed by addition of 50 μ l Z-buffer with
0.4% (w/v) SDS and incubated at 30°C for 10 min. 50 μ l of Z-buffer with 4mg/ml O-
nitrophenyl- β -D-galactopyranoside were added and the optical density at 420nm was

recorded automatically every 15s over a period of 60min. Specific β -gal activity was calculated from the V_{\max} as in Miller (*Op Cit.*).

5 **Example 1:** NifA chimera with heterologous DNA binding domains activate transcription but only from promoters with a cognate recognition site

To investigate in what way transcription activation by NifA was dependent on the NifA DNA binding domain (DBD) and on native nif promoter structure, we prepared NifA-
10 chimeras in which NifA DNA binding domain (DBD) had been replaced by heterologous DBDs of diverse structural architectures. Initially we explored DBDs which, like the NifA wild type (wt) DBD bind to symmetrical DNA recognition sequences such as the basic leucine zipper (bZIP) DBD of the yeast transcription factor GCN4, the Zn-finger domain of the human estrogen receptor DNA binding domain (ERDBD) and determined their
15 capacity to activate transcription of a lacZ reporter gene in vivo from a hybrid nifH promoter, in which the NifA UAS had been deleted and replaced by recognition sites for the heterologous DBDs.

In order to simplify comparison of transcription activation by NifA chimeras with
20 activation by wt NifA, all reporter constructs had a single DNA recognition site. The wt nifH promoter UAS contains three bona fide NifA recognition sites. Deletion of the two sites more distal to the promoter, however, did not appear to reduce transcription activation in our reporter under conditions tested.

25 Transcription activation by NifA-chimeras was specific in that they only activated lacZ expression from hybrid-promoters bearing their cognate recognition sequences but not from control reporter constructs bearing wild type UAS or a non-cognate site (Fig. 3). In analogy to wt NifA the presence of two or more recognition sites (in phase, see below) did not increase activation by the Nif-GCN4 chimera.

30

Activity was also dependent on the phasing of the recognition site with respect to the promoter: when the symmetric ATF/CREB recognition site for GCN4 was offset in

increments of 1 bp, optimal activity was observed when the ATF/CREB was centred on the same bp as the symmetric wt UAS. Presumably efficient contact with the RNA Pol holoenzyme requires that the activator be bound on the right face of the DNA.

- 5 Transcription activation by NifA-chimeras appears to preserve fine specificity of isolated DBDs. Wild-type GCN4 binds with equal affinity to the symmetric ATF/CREB site as well as to the pseudo-symmetric AP-1 site. Indeed, the NifA-GCN4 chimera showed identical levels of transcription activation in reporter constructs with either of these sites (Fig. 3). A NifA-ERDBD chimera showed strong activity on a reporter with its cognate
10 ERE site but no activity above background levels with reporters bearing the similar GRE recognition site for the closely related glucocorticoid receptor DBD (Fig. 4).

Example 2: Coexpression of wild-type NifA with NifA-chimeras boosts specific transcription activation by NifA chimeras in a specific and DNA independent manner

15

The level of transcription activation by the NifA-GCN4 and NifA-ERDBD chimeras was lower (ca. 10%) than for wt NifA. However, near wt levels of activity (up to 80%) were reached when wt NifA was coexpressed within the same cell as a "coactivator".

- 20 The coactivation was independent of DNA binding, as NifA variants in which the DBD had been deleted (NifA Δ C) was found to be just as active as wt NifA. On the other coexpression of an isolated NifA central domain (both the DBD as well as the N-terminal sensor domain deleted (NifA Δ NC)) failed to coactivate. NifA derived from different species showed greatly variable efficiencies as coactivators. NifA variants from *K.*
25 *pneumoniae* (NifA Kp, NifA Δ C Kp) were almost three times as effective as NifA, while NifA variants from *Rhizobium* (NifA Rh1, NifA Rh2) were poorly active as coactivators (Fig. 5).

- The coactivator effect was found to enhance only specific transcriptional activation and not
30 background levels of transcription from promoters with non-cognate recognition sites. We therefore constructed an *E. coli* strain, expressing NifA Δ C Kp (the *K. pneumoniae* NifA with its DBD deleted) from a weak promoter (phoB) from the chromosome (TG1: Δ K).

The coactivation effect has analogies in eukaryotic transcription. for example the enhancer Sp1, in which isolated Sp1 activation domains can stimulate transcriptional activation by the DNA binding-form of Sp1, a phenomenon termed "superactivation".

5

Example 3: Tethering of NifA chimera at the UAS is sufficient for activation, but strong activation requires correct positioning

10 We also investigated transcription activation by NifA-chimeras with asymmetrical recognition sites such as the classic Zn-finger Zif268 as well as the DBD from p53.

Both NifA-Zif268 and NifA-p53 chimeras activated transcription, but only at low levels (2 - 5-fold above the background). However, when the Zif recognition site was duplicated, to
15 give a symmetric palindromic site transcription activation increased substantially. Non-palindromic duplication of the recognition site in tandem did not increase activation.

Thus while simple tethering is sufficient for some activation, only bipartite binding appears to give a strong activation. Presumably, tethering only leads to an approximate
20 positioning of the activation domain with respect to the RNA polymerase holoenzyme, thereby reducing the likelihood of a productive interaction.

Example 4: Selection of active NifA-chimeras by lac complementation

25

Using expression of the lac operon (lacZYA) from our reporter construct as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δ lac strain, with lactose as the only carbon source. Initially we spiked populations of NifA-ERDBD with NifA-GCN4 at the ratios 1/10⁴,
30 1/10⁶ in the presence of the GCN4 cognate reporter ATF/CREB-nifH and grew populations overnight in minimal medium supplied with lactose. Pre- and post selection populations were scored by plating on MacConkey-lactose plates as well as by PCR

screening. The results are summarised in Table 1. Selection factors of up to 10.000 -fold per round were observed. .

Table 1: Selection factors for Nif selection by lac complementation

5

| NifGCN4/NifERDBD | Selection factor |
|-------------------|------------------|
| 1/10 ⁴ | 40 fold |
| 1/10 ⁵ | 40 fold |
| 1/10 ⁶ | 200 fold |
| 1/10 ⁷ | 4000 fold |

Example 5: Selection of active NifA-chimeras by flow cytometry.

- 10 Expression of β -galactosidase (lacZ) as the read-out of transcription activation allows the selection of active NifA-chimera on the basis of metabolic complementation of a Δ lac strain, grown on lactose as the only carbon source. However, metabolic selection predisposes the system to the generation of false positives. Presumably, the prolonged growth under metabolic selection selects for mutant promoters, active in the absence of a
- 15 cognate enhancer.

We have observed that that this only occurs for library sizes exceeding 10⁶. Indeed, others have found (using a related bacterial two-hybrid system) that it is not possible to retrieve positive clones from dilutions higher than 1/10⁶ by metabolic lac selection (G. Karimova, et al., (1998) Proc Natl Acad Sci USA 95, 12532-7). As it is well known that bacteria can develop a mutator phenotype under adaptive stress (P. D. Sniegowski, P. J. Gerrish, R. E. Lenski. (1997) *Nature* 387, 703-5), we conclude that it is preferable to separate the selection from the amplification (growth) step in order to reduce the likelihood of revertants.

25

We thus replaced lacZ with the *Aequorea victoria* green fluorescent protein (EGFP, F64L, S65T, ex488 nm, em527nm (the Clontech variant pGFPmut3 1, S65E, S72A, ex501nm, em511nm FACS optimised variant was also tried, but found inferior) as the reporter gene. GFP has the advantage that cells can be grown first and then separated on the basis of
5 fluorescence using fluorescence activated cell sorting (FACS).

We prepared a trial library of mutant GCN4 bZIP DBDs (library size 10^8) in which 5 key residues (Asn235, Ala238, Ala239, Ser242, Arg243) of GCN4 interacting with DNA were randomised and selected it against a GFP hybrid reporter with the cognate ATF/CREB
10 site. Library populations were grown overnight at 34°C in non-fluorescent medium NFM (minimal medium supplied with 2% glucose, 0.2% casaminoacids, 12 ng/ml L-Trp). For FACS (Cytomation Mofo, 488 nm Laser, FL-1 530/40 filter) an 1 ml aliquot was diluted 10X in NFM and the top 1% fluorescent cell population was sorted into a 96 well plate at 1 cell per well, and grown up overnight at 34°C. Cell fluorescence of the grown up clones
15 was measured by using a SPECTRAmax^RGEMINI Dual-Scanning Microplate Spectrofluorometer (Molecular Devices), ex480, em520, (cut-off 515 nm). Plasmids from fluorescent wells were sequenced afterwards. Pre- and post selection populations were also scored by PCR screening as well as by plating on min glu (M9 Minimal medium + glucose) plates and visualised using fluorescence microscope.

20 10^5 cells were sorted in total, from which 219 cells were in the top 1% fluorescent population and 132 of which were captured to the 96-well plates. 13 cells from these were fluorescent. Selected positives were checked by separating the mutant GCN4-bZIP DBD expressor plasmids, and re-transforming them together with cognate and non-cognate
25 reporter plasmids. None of the selected positives gave a fluorescent signal when combined non-cognate reporter plasmids, but all were fluorescent when combined with the ATF/Creb cognate reporter plasmid (which did not produce any fluorescence when transformed on its own).

30 This indicates that GFP selection indeed avoids the isolation of false positives. Furthermore, when the library was checked prior to FACS sorting no fluorescent clones

were identified when plating $>10^7$ cells. 1/10 clones plated post selection were fluorescent, suggesting a selection factor in a single round in excess of 10^9 -fold.

5 All publications mentioned in the above specification are herein incorporated by reference. All database sequences denoted by accession or gi numbers are likewise incorporated by reference.

10 Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following
15 claims.

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Claims

1. A method for detecting a protein-nucleic acid interaction between a nucleic acid molecule and a protein molecule, comprising the steps of:
 - 5 a) providing one or more hybrid $\sigma 54$ activator proteins comprising a heterologous nucleic acid binding sequence and a constitutively active $\sigma 54$ transcription activating domain;
 - b) providing one or more nucleic acid molecules comprising a binding site for the nucleic acid binding sequence and a binding site for $\sigma 54$ RNA polymerase (RNAP), which
10 directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain; and
 - c) detecting expression of the reporter gene.
2. A method according to claim 1, comprising providing a repertoire of hybrid $\sigma 54$
15 activator proteins, said repertoire comprising a plurality of different nucleic acid binding sequences.
3. A method according to claim 1, comprising providing a repertoire of hybrid nucleic acid molecules, said repertoire comprising a plurality of different binding sites for the
20 nucleic acid binding sequence.
4. A method according to claim 1, comprising providing both a repertoire according to claim 2 and a repertoire according to claim 3.
- 25 5. A method for detecting a protein-protein interaction, comprising the steps of:
 - a) providing a first hybrid protein comprising a nucleic acid binding sequence and a first polypeptide sequence bait;
 - b) providing a second hybrid protein comprising a prey polypeptide sequence and constitutively active $\sigma 54$ transcription activating domain;
 - 30 c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a

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reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the first and second hybrid proteins together with the nucleic acid molecule such that the prey and bait polypeptide sequences may bind, thereby forming a hybrid protein comprising both a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain; and

e) detecting expression of the reporter gene.

6. A method according to claim 5, comprising providing a repertoire of first hybrid proteins, said repertoire comprising a plurality of bait polypeptides.

7. A method according to claim 5, comprising providing a repertoire of second hybrid proteins, said repertoire comprising a plurality of prey polypeptides.

8. A method according to claim 5, comprising providing a repertoire of first hybrid proteins and a repertoire of second hybrid proteins, said repertoires comprising a plurality of bait and prey polypeptides.

9. A method for screening a repertoire of candidate DNA-bending polypeptides, comprising the steps of:

a) providing a repertoire of candidate polypeptide factors with potential to induce bending of DNA;

b) providing a $\sigma 54$ activator protein comprising a nucleic acid binding sequence and a $\sigma 54$ transcription activating domain;

c) providing a nucleic acid molecule comprising a binding site for the nucleic acid binding sequence and binding site for $\sigma 54$ RNAP which directs the expression of a reporter gene and leads to upregulation thereof in response to activation by the $\sigma 54$ transcription activating domain;

d) incubating the repertoire and $\sigma 54$ activator together with the nucleic acid molecule in a IHF⁻ host cell, such that $\sigma 54$ activator and the nucleic acid molecule may interact, and transcription activated from the $\sigma 54$ RNAP binding site in a manner dependent on DNA bending by the polypeptide factors; and

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gb|AA99303.1| (L43064) regulatory protein [*Pseudomonas aeruginosa*];

- gb|AAB91397.1| (AF033203) nifaii protein [Rhodobacter capsulatus];
 gb|AAC05586.1| (AF006075) regulatory protein [Bacillus subtilis];
 gb|AAC37124.1| (L81176) fleq [Pseudomonas aeruginosa];
 gb|AAC45640.1| (AF010585) putative sigma 54 activator [Caulobacter crescentus];
 5 gb|AAC46367.1| (AF014113) two-component response regulator [Vibrio cholerae];
 gb|AAD34591.1|AF145956_1 (AF145956) transcriptional activator nifa [Rhodospirillum
 rubrum];
 gb|AAD38416.1| (AF155934) nifa [Alcaligenes faecalis];
 gb|AAF28395.1| (AF069392) flam [Vibrio parahaemolyticus];
 10 gb|AAF33506.1| (AF170176) Salmonella typhimurium transcriptional regulatory protein;
 gb|AAF61932.1| (AF230804) sigma-54 activator protein ActI [Myxococcus xanthus];
 gb|AAF85342.1|AE004061_7 (AE004061) two-component system, regulatory protein
 [Xylella fastidiosa];
 gb|AAF94676.1| (AE004230) sigma-54 dependent response regulator [Vibrio cholerae];
 15 gb|AAF95280.1| (AE004286) sigma-54 dependent response regulator [Vibrio cholerae];
 gb|AAF96095.1| (AE004358) sigma-54 dependent transcriptional regulator [Vibrio
 cholerae];
 gb|AAG01527.1|AF288483_1 (AF288483) nifa [Azospirillum brasilense];
 pir|A48291 ornithine decarboxylase inhibitor - Escherichia coli;
 20 pir|B49940 nitrogen regulator I homolog - Escherichia coli;
 pir|C70320 transcription regulator nifa family - Aquifex aeolicus;
 pir|C70396 transcription regulator ntrc family - Aquifex aeolicus;
 pir|C70454 transcription regulator ntrc family - Aquifex aeolicus;
 pir|D70315 transcription regulator ntrc family - Aquifex aeolicus;
 25 pir|H69581 transcription activator of acetoin dehydrogenase operon acor - Bacillus
 subtilis;
 pir|I39719 nitrogen regulatory protein - Agrobacterium tumefaciens;
 pir|JC5471 regulatory protein nifa - Azospirillum lipoferum;
 pir|T08624 probable ntrc-type response regulator - Eubacterium acidaminophilum;
 30 sp|P03027|NIFA_KLEPN NIF-SPECIFIC REGULATORY PROTEIN;
 sp|P09570|NIFA_AZOVI NIF-SPECIFIC REGULATORY PROTEIN;
 sp|P12627|VNFA_AZOVI NITROGEN FIXATION PROTEIN VNFA;

12-11-2001

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- sp|P14375|HYDG_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
sp|P21712|YFHA_ECOLI HYPOTHETICAL 49.1 KD PROTEIN IN GLNB-PURL
INTERGENIC REGION (ORFXB);
sp|P24426|NIFA_RHILT NIF-SPECIFIC REGULATORY PROTEIN;
5 sp|P25852|HYDG_SALTY TRANSCRIPTIONAL REGULATORY PROTEIN HYDG;
sp|P27713|NIFA_1IERSE NIF-SPECIFIC REGULATORY PROTEIN;
sp|P30667|NIFA_AZOB R NIF-SPECIFIC REGULATORY PROTEIN;
sp|P38035|RTCR_ECOLI TRANSCRIPTIONAL REGULATORY PROTEIN RTCR;
sp|P54929|NIFA_AZOLI NIF-SPECIFIC REGULATORY PROTEIN;
10 sp|P56266|NIFA_KLEOX NIF-SPECIFIC REGULATORY PROTEIN;
sp|Q06065|ATOC_ECOLI ACETOACETATE METABOLISM REGULATORY
PROTEIN ATOC (ORNITHINE/ARGININE;
sp|Q46802|YGEV_ECOLI HYPOTHETICAL SIGMA-54-DEPENDENT
TRANSCRIPTIONAL REGULATOR IN;
15 sp|Q53206|NIFA_RHISN NIF-SPECIFIC REGULATORY PROTEIN; and
sp|Q9ZIB7|TYRR_ERWHE TRANSCRIPTIONAL REGULATORY PROTEIN TYRR.

15. A method according to any one of claims 1 to 14, wherein the $\sigma 54$ transcriptional
activator is the NifA transcriptional activator or the PspF transcriptional activator.
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16. A method according to any one of claims 1 to 14, wherein the hybrid $\sigma 54$
transcriptional activator is NifA and activation resulting from NifA- $\sigma 54$ RNAP interaction
is enhanced by the coexpression of wild-type or mutant NifA.
- 25 17. A method according to claim 16, wherein the hybrid $\sigma 54$ transcriptional activator is
NifA from *Azotobacter vinelandii*, and the wild-type or mutant NifA is NifA from
Klebsiella pneumoniae.
18. A method according to any preceding claim, wherein the nucleic acid molecule
30 comprises a binding site for a factor which induces DNA bending.
19. A method according to claim 18, wherein the factor is integration host factor (IHF).

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20. A method according to any one of claims 1 to 17, wherein the nucleic acid molecule comprises DNA that is intrinsically bent.
- 5 21. A method according to any preceding claim, wherein the nucleic acid molecule comprises a *nifH* promoter from *A. vinelandii* driving a reporter gene.
22. A method according to any preceding claim, wherein the reporter gene is selected from the group consisting of metabolic markers such as the lac operon (*lacZ*, *lacY* and
10 *lacA*); proteins conferring a fluorescent phenotype, such as GFP; proteins conferring antibiotic resistance, such as Zeo; and proteins conferring another selectable property.
23. A method according to any preceding claim, which is carried out in the presence of a compound which modifies protein-protein or protein-DNA interaction.
- 15 24. A method according to claim 22, wherein the compound is selected from the group consisting of molecules which alter the structure of the DNA-binding protein; molecules which alter the structure of DNA; and molecules which modify protein-protein interactions.
- 20 25. A method according to any preceding claim, which is carried out *in vivo*.
26. A method according to claim 25, wherein the *in vivo* host is *E. coli*.
- 25 27. A method according to any one of claims 1 to 24, which is carried out *in vitro*.

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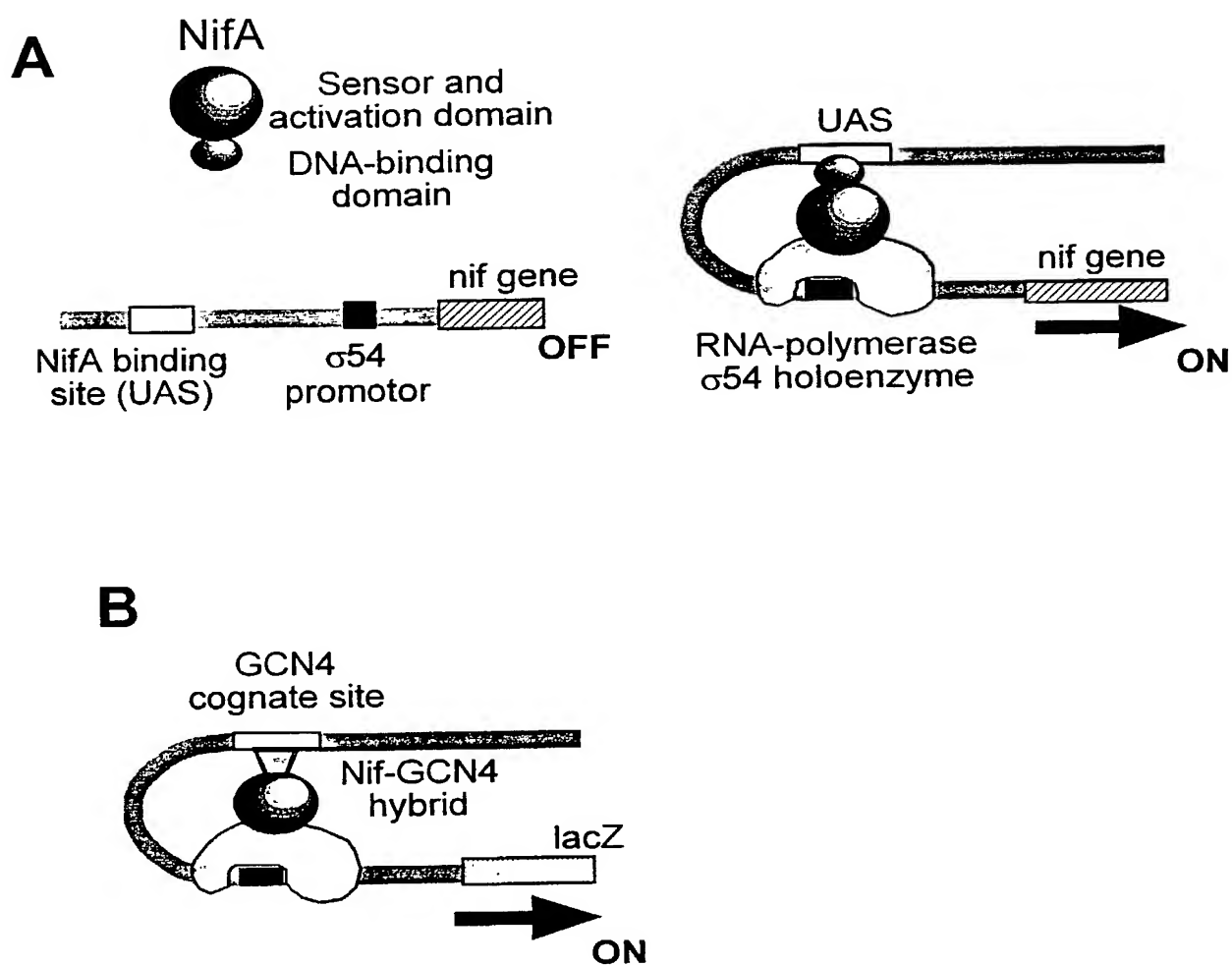
(54) Title: **SELECTION SYSTEM**

(57) Abstract: A genetic selection system is described based on hybrid $\sigma 54$ RNAP activator proteins. The invention permits the detection and screening for protein:DNA and protein:protein binding events.

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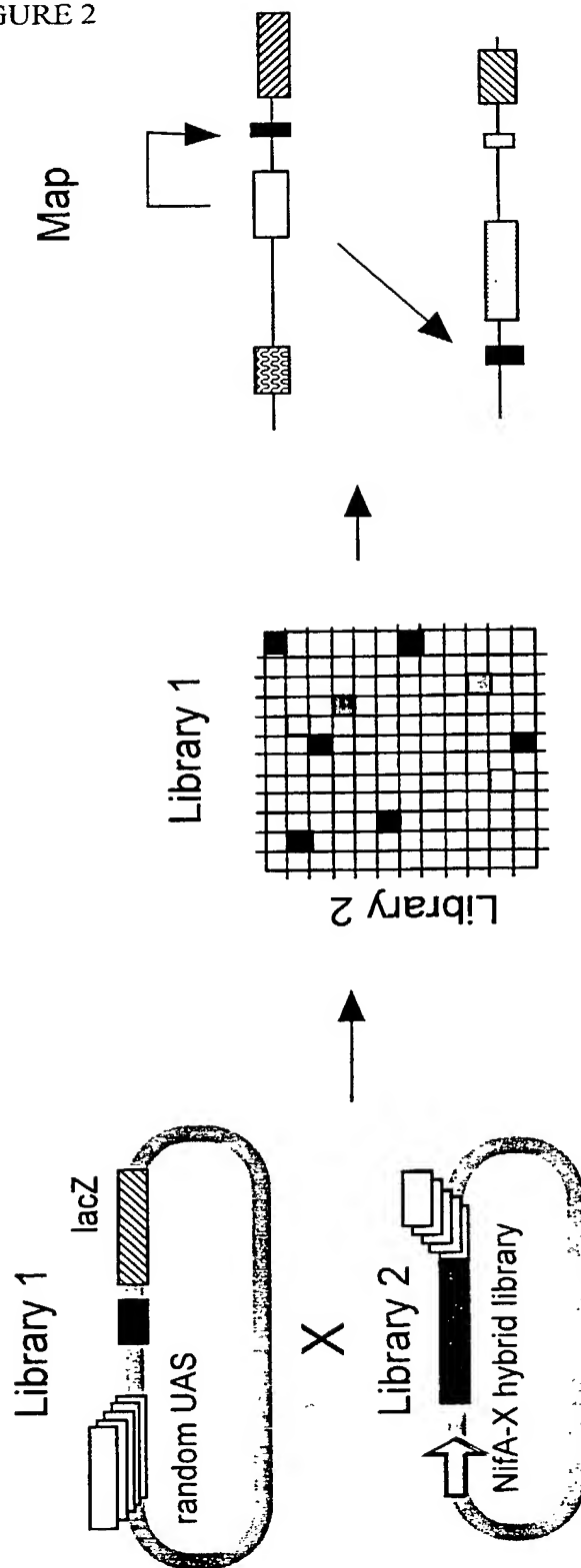
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FIGURE 1



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FIGURE 2



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Figure 3

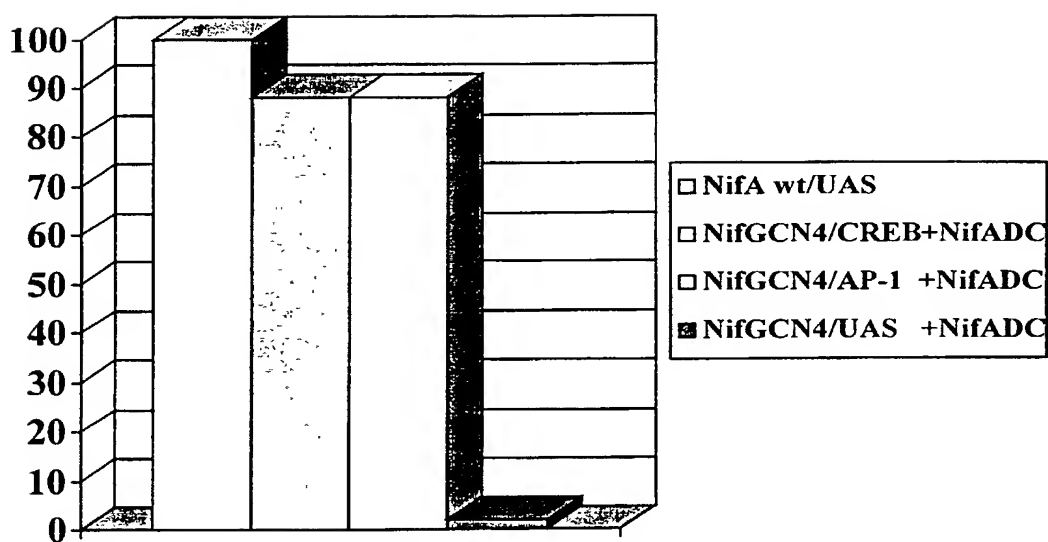
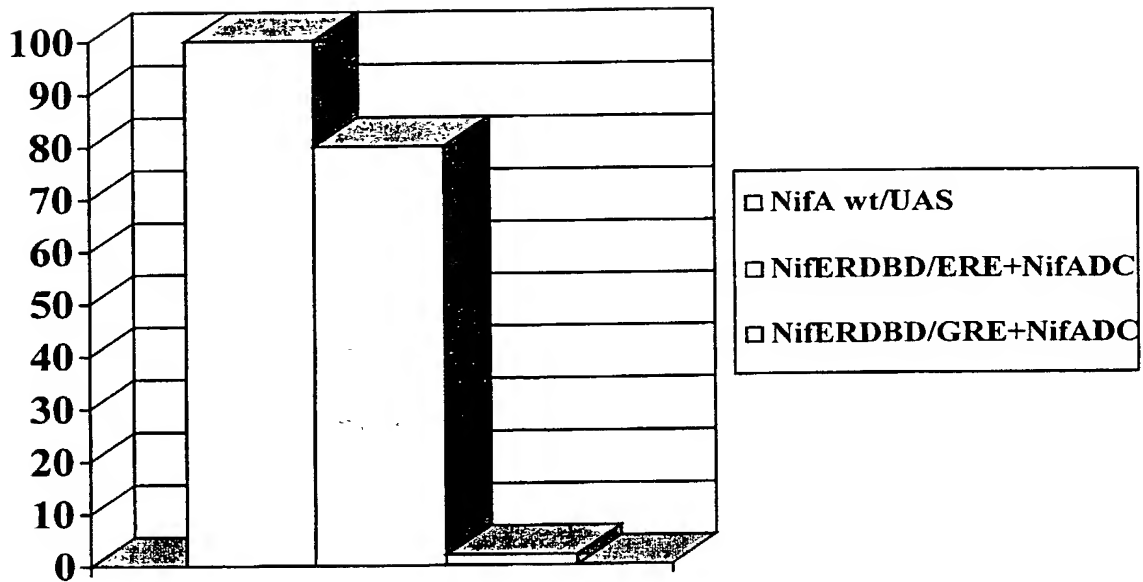
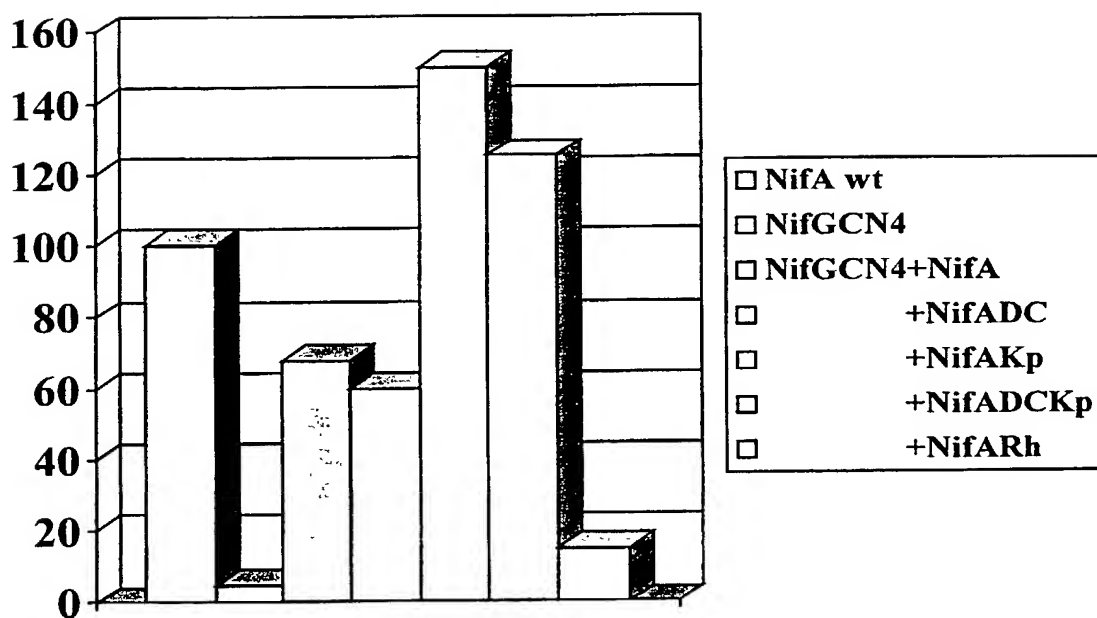


Figure 4



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Figure 5



PATENT/OFFICIAL
Docket No.: 109312.122US1

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office and citizenship are as stated below next to my name,

I believe I am a first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below), of the subject matter claimed and for which a patent is sought on the invention entitled:

SELECTION SYSTEM,

the specification of which

☐ is attached hereto OR
☒ was filed on March 8, 2002 as Application Serial No. 10/070,667.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is known to me to be material to patentability in accordance with Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

| Prior Foreign Application(s): | | | Priority Claimed | |
|-------------------------------|----------------|-----------------------------|------------------|-----------|
| <u>Number</u> | <u>Country</u> | <u>Day/Month/Year filed</u> | <u>Yes</u> | <u>No</u> |
| 9921155.9 | GB | 08 September 1999 | X | |

I hereby claim the benefit under 35 USC 119(e) of any United States provisional application(s) listed below.

Prior Provisional Application(s):
Application Number Filing Date

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| Prior U. S. Non-Provisional Application(s): | | |
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